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(54) Title: LIPOSOMAL NUCLEOSIDE ANALOGUES FOR TREATING AIDS

(57) Abstract

A composition is disclosed for use in treating acquired immune deficiency syndrome (AIDS) and related retroviral infections. This composition consists of a phosphorylated nucleoside analogue which is encapsulated in a liposome. 5'-mono-phosphate derivatives of dideoxydinucleoside analogues such as AZT, ddC and ddA are encapsulated in liposomes in a manner which prevents or substantially reduces leakage, resulting in reduced toxic side effects of these drugs and enhanced inhibition of replication of HIV or related viruses present in monocyte/macrophages and other infected cells.

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LIPOSOMAL NUCLEOSIDE ANALOGUES FOR TREATING AIDS

BACKGROUND OF THE INVENTION

Field of the Invention

The present invention relates generally to the treatment of viral infections with nucleoside analogues. More particularly, the present invention relates to the encapsulation of modified antiviral nucleoside analogues in liposomes to enhance the effectiveness of the analogues when administered to mammals.

This invention was made with Government support under Grant No.: GM-24979 from the National Institutes of Health to the University of California and the Veterans Administration. The Government has certain rights in this invention.

Description of Related Art

The publications and other reference materials referred to herein to describe the background of the invention and to provide additional detail regarding its practice are hereby incorporated by reference. For convenience, the reference materials are numerically referenced and grouped in the bibliography appended at the end of this specification.

There has been a great deal of interest in recent years in the use of nucleoside analogues to treat viral infections. The antiviral nucleoside analogues are designed to inhibit viral functions by preventing the synthesis of new DNA by viral reverse transcriptase during viral replication. Nucleosides are the precursors of DNA or RNA. A nucleoside consists of a pyrimidine or purine base which is linked to a five-carbon sugar.

During DNA synthesis, free nucleoside triphosphates

(nucleosides with three phosphate groups attached) react with the end of a growing DNA chain. The reaction involves the linking of the phosphate group at the 5′ position on the incoming nucleoside triphosphate with the hydroxyl group at the 3′ position of the sugar ring on the end of the forming DNA chain. The other two phosphate groups are freed during the reaction thereby resulting in the addition of a nucleotide to the DNA chain.

Nucleoside analogues are compounds which mimic the naturally occurring nucleosides sufficiently so that they are able to participate in viral DNA synthesis. However, the antiviral nucleoside analogues have strategically located differences in chemical structure which inhibit the viral enzyme reverse transcriptase or which prevent further DNA synthesis once the analogue has been attached to the growing DNA chain. Azidothymine (AZT), dideoxycytidine, dideoxyadenosine, acyclovir, ribavirin and vidarabine are examples of nucleoside analogues which have been under investigation or have been found effective in disrupting viral DNA or RNA synthesis (1).

Acquired immune deficiency syndrome (AIDS) has been referred to as the first great pandemic of the second half of the 20th century (2). AIDS is caused by the human immune deficiency virus (HIV). There is no effective cure for AIDS at the present time. Dideoxynucleoside analogues such as AZT are the most potent agents currently known, but in a recent human trial, serious toxicity was noted consisting of anemia (24%) and granulocytopenia (16%) (37, 38).

HIV infects cells bearing the CD4 (T4) surface antigen such as CD4 helper lymphocytes as well as CD4 monocytes and macrophages. The infection of CD4 lymphocytes (3-5) results in a cytolytic infection and contributes to the progressive immunodeficiency of HIV

infection (6, 7). More recently, CD4 monocyte/macrophages have been shown to be infected in vitro (8, 9), and in vivo (10-15). Infection of monocyte/macrophages may also contribute to immunodeficiency and to the pathogenesis of HIV induced encephalopathy. In addition, these cells may serve as a reservoir for the virus because HIV replication in monocyte/macrophages appears to be more prolonged and less cytolytic than in lymphocytes. (8, 9, 13).

It would be desirable to provide a means for administering AZT and other dideoxynucleosides in a manner such that the toxic side effects of these drugs are reduced. Further, it would be desirable to provide selective targeting of the dideoxynucleoside to monocyte/macrophages to enhance the efficiency of the drug against viral infection in this group of cells.

In 1965 Alex Bangham and coworkers discovered that dried films of phosphatidylcholine spontaneously formed closed bimolecular leaflet vesicles upon hydration. They used these dispersions to study the capture and diffusion of cations (18). Eventually these structures came to be known as liposomes. Subsequently many potential uses for liposomes were suggested and by 1976 over 60 substances had been reported to become encapsulated in liposomes and a wide variety of uses for liposomes in medicine had been suggested including oral administration of liposomal insulin, selective targeting of liposomes to organs, replacement of missing enzymes and genetic material by liposomal carriers (19).

Many of the above goals have proved elusive. Selective targeting to most organs now seems virtually unattainable because of the tightness of the vascular system; only organs having sinusoids or fenestrated endothelium seem to be possible targets (20, 21). Replacement of deficient enzymes or genetic material has also been difficult because of the relatively rapid

removal of liposomes from the vascular compartment and because fusion of liposomes with cell surface membranes seems not to occur readily (20).

As noted in a recent review by Marc Ostro there are many promising uses of liposomes which are nearing the clinical arena (22). For example, liposomal antimonial drugs are several hundred fold more effective than the free drug in treating leishmaniasis as shown independently by Black and Watson (23) and Alving et al (24). Liposome-entrapped amphotericin B appears to be more effective than the free drug in treating immunosuppressed patients with systemic fungal disease (25, 26). Other uses for liposome encapsulation include restriction of doxorubicin toxicity (27) and diminution of aminoglycoside toxicity (22).

As previously mentioned it is now thought that macrophages are an important reservoir of HIV infection (28, 29 and 30) and that macrophages are also a primary site of liposome uptake (20, 21). Accordingly, it would be desirable to utilize liposomes to enhance the effectiveness of antiviral nucleoside analogues in treating AIDS.

Attempts have been made to incorporate nucleoside analogues, such as iododeoxyuridine (IUDR), acylovir (ACV) and ribavirin into liposomes for treating diseases other than AIDS (16, 17). However, these attempts have not been entirely satisfactory because these relatively small nucleoside analogues tend to rapidly leak out of the liposome (16, 17) resulting in decreased targeting effectiveness.

SUMMARY OF THE INVENTION

In accordance with the present invention a new treatment for AIDS is disclosed in which antiviral nucleoside analogues are trapped within liposomes in a manner which prevents or substantially reduces leakage.

This reduction in leakage provides reduced toxicity and the use of liposomes ensures that a greater amount of the antiviral nucleoside analogue reaches the macrophages to thereby increase the effectiveness of these drugs against the HIV infection present in such cells. The analogue of the neucleoside encapsulated in liposomes further enhances the antiviral effect of the formulation by providing a prephosphorylated antivral nucleoside, bypassing an enzymatically deficient step in macrophages which would otherwise greatly limit the antiviral activity of the drug itself (43).

The present invention is based on the discovery that leakage of nucleoside analogues from liposomes is greatly reduced if the analogues are converted to phosphate derivatives prior to encapsulation in liposomes. Conversion of the nucleoside analogues to their respective phosphate derivatives is believed not only to prevent leaking of the analogues from the liposomes, but also to increase the effectiveness of the analogues against HIV-infected macrophage cells. As previously mentioned, nucleosides are phosphorylated to the triphosphate form prior to attachment to the growing DNA or RNA chain. Macrophages have been found to have diminished deoxynucleoside kinase activities and a reduced ability to phosphorylate nucleosides. Accordingly, free nucleoside analogues (i.e., non-phosphorylated analogues), such as AZT, ddC or ddA are not particularly effective against HIV present in macrophage cells (43). However, the phosphorylated analogues of the present invention are more effective when presented by the lipsomal delivery system because they by-pass the metabolic block caused by the lack of deoxynucleoside kinase activity in macrophages.

The above-discussed features and attendant advantages of the present invention will become apparent as the invention becomes better understood by reference

to the following detailed descriptions.

DETAILED DESCRIPTION

The present invention basically involves the encapsulation of phosphorylated antiviral nucleoside analogues within liposomes for use in treating viral infections. Although this invention has applications to a wide variety of nucleoside analogues which may be used to treat various viral infections, the following description will be in the context of the treatment of patients suffering from Acquired Immune Deficiency Syndrome (AIDS) and related retroviral infections such as AIDS-related complex (ARC), asymptomatic infections with HIV-1 and HIV-2 and malignant (lymphoma) or neurologic (tropical spastic paralysis) complications of HTLV-1 or HTLV-2.

Human immunodeficiency virus (HIV, previously referred to as HTLV-III/LAV) is the etiologic agent of AIDS. As extrapolated from serological surveys, over 1,000,000 Americans have been infected since the virus was introduced into the United States in the late 1970's. By now it is estimated that as many as two million people may be infected in the United States. Although the majority of these seropositive individuals are asymptomatic, most, if not all, remain persistently infected and thus constitute a pool of potential transmitters of infection. A significant fraction of these individuals have lymphadenopathy and other symptoms, and a smaller proportion, (a few percent per year) progress to AIDS with its grim prognosis. This minority now numbers over 35,000 in the United States alone and the number is doubling approximately yearly.

At a cellular level, HIV infects the CD4 (T4) helper lymphocyte resulting in the death of these cells. Depletion of CD4 helper lymphocytes makes the host vulnerable to certain well described opportunistic

infections and malignancies. HIV binds to the CD4 receptor of lymphocytes by forming a complex between the 110K viral surface glycoprotein (gp110) and the CD4 antigen (32, 32). Subsequently, the virus is thought to enter the cell by endocytosis as suggested by the finding that productive infection is blocked by NH_4Cl and amantadine pretreatment of the cells. After presumed acid-mediated fusion with the endosome membrane the viral reverse transcriptase and RNA gain access to the cytoplasm and ultimately the viral genetic material gains access to the cell genome. As recently reviewed by Yarchoan and Broder (33) there are at least 9 steps in HIV replication which may provide targets for therapeutic intervention. At present, inhibition of viral reverse transcriptase represents the most promising avenue of antiretroviral therapy.

Recently it has been demonstrated that cells other than the CD4 helper lymphocyte become infected with HIV (28, 29, 30). The virus has been shown to replicate in B cells, promyelocytes and monocytes. It also has been shown that mononuclear phagocytes isolated from brain and lung harbored HIV and normal peripheral blood macrophages produced large quantities of virus. Furthermore, human alveolar macrophages and brain macrophages harbor HIV and the viral cytopathic effects on these cells are much less than that observed in HIV-infected helper T4 lymphocytes. It has been proposed that HIV-infected macrophages and monocytes may serve as a reservoir for virus and that this may be a mechanism for viral persistence and dissemination in the infected host (29, 30).

The present invention utilizes the affinity of macrophages for liposomes as a vehicle for directing liposome encapsulated antiviral nucleoside analogues at macrophages, monocytes and any other infected cells which may take up liposomes. Further, phosphorylation

of the nucleoside analogue prior to encapsulation provides advantages in that: 1) the nucleoside analogue is prevented from leaking out of the liposome; and 2) the metabolic block associated with the inability of macrophages to phosphorylate free nucleosides is overcome. Finally, liposomal encapsulation of the phosphorylated nucleoside is essential to prevent hydrolysis of the phosphate ester by plasma enzymes such as alkaline phosphatase, phosphodiesterases or 5'-nucleotidases.

The nucleoside analogues can be any of the known analogues used for treating AIDS including 3'-azido-3'-deoxythymidine (azidothymidine or AZT), 2',3'-dideoxycytidine (dideoxycytidine or ddC), 2'3'-dideoxyadenosine (dideoxyadenosine or ddA), ribavirin or any other suitable dideoxynucleoside analogue. AZT is a preferred analogue.

The analogue is phosphorylated according to conventional procedures such as the phosphorous oxychloride method of Toorchen and Topal (34). The preferred modified analogue is the 5'-monophosphate. Since AZT and other dideoxynucleosides have only the 5'-hydroxyl, only the 5'-monophosphate is formed during phosphorylation. Alternatively, the nucleoside 5'-monophosphate thioester is also effective. Diphosphate and triphosphate analogues of antiviral nucleosides are also effective, however, these diphosphate or triphosphate analogues tend to be less stable than the monophosphate and may be hydrolyzed gradually back to the monophosphate derivative in aqueous solution.

After phosphorylation, the nucleoside analogue is encapsulated in liposomes. The encapsulation can be carried out according to well known liposome encapsulation procedures such as sonication and extrusion. Suitable conventional methods of encapsulation include but are not limited to those disclosed by Bangham et al

(18), Olson et al (39), Szoka and Papahadjapoulos (40), Mayhew et al (41), Kim et al (42), Mayer et al (36) and Fukunaga et al (35).

The liposomes can be made from any of the conventional synthetic or natural phospholipid liposome materials including phospholipids from natural sources such as egg, plant or animal sources such as phosphatidylcholine, phosphatidylethanolamine, phosphatidylglycerol, sphingomyelin, phosphatidylserine or phosphatidylinositol. Synthetic phospholipids may also be used, such as, but not limited to, dimyristoylphosphatidylcholine, dioleoylphosphatidylcholine, dipalmitoylphosphatidylcholine, distearoylphosphatidylcholine, dilauroylphosphatidylethanolamine, dimyristoylphosphatidylcholine, dipalmitoylphosphatidylcholine, dioleoylphosphatidylethanolamine and distearoylphosphatidylethanolamine. Other additives such as cholesterol or other sterols, glycolipids, cerebrosides, gangliosides, sphingolipids, glucopsychosine, or psychosine can also be added as is conventionally known. The relative amounts of phospholipid and additives used in the liposomes may be varied if desired. The preferred ranges are from about 80-99 mole percent phospholipid and 1 to 20 mole percent psychosine or other additive. Cholesterol may be used in amounts ranging from 0 to 50 mole percent. The relative amounts of antiviral nucleoside analogue entrapped in liposomes can be varied with the concentration of entrapped analogue in the liposome aqueous compartment ranging from about 0.001 mM to about 300 mM.

The liposome entrapped phosphorylated nucleoside analogue is administered to patients by any of the known procedures utilized for administering liposomes. The liposomes can be administered intravenously, intraperitoneally or intramuscularly as a buffered aqueous solution. Any pharmaceutically acceptable aqueous

buffer may be utilized so long as it does not destroy the liposome structure or the activity of the encapsulated phosphorylated nucleoside analogue. One suitable aqueous buffer is 150 mM NaCl containing 5mM Na-Phosphate with a pH of about 7.4; other physiological buffered salt solutions may also be used.

The dosage may vary depending upon the extent and severity of the infection. Dosage levels of encapsulated phosphorylated nucleoside analogue should be such that about 0.001 mg/kilogram to 1000 mg/kilogram be administered to the patient on a daily basis.

Examples demonstrating the reduction in leakage of phosphorylated nucleoside analogues from liposomes are as follows:

Initial studies were carried out with [^{3}H]thymidine and [^{14}C]thymidine-5'-monophosphate, 28.6x10^{6}DPM of [^{3}H]thymidine and 2.510^{6}DPM of [^{14}C]thymidine-5'-monophosphate [^{14}C]TMP in 2.0 ml RPMI buffer was added to a thin film of egg phosphatidylcholine/cholesterol (molar ratio 2:1). After vortexing and swelling for 10 min the suspension was sonicated for 20 min and passed over a Sepharose 4B column as described by Fukunaga et al, (35). Table 1 shows the DPM (and %) of each compound entrapped in multilamellar (MLV) and small unilamellar vesicles (SUV). 1.1% of the [^{14}C]TMP was retained in MLV versus only 0.1% of the [^{3}H]thymidine. In SUV, 5% of the [^{14}C]TMP was trapped versus only 0.4% of the [^{3}H]thymidine.

TABLE 1

RETENTION OF RADIOLABELED NUCLEOSIDES IN PC/CHOLESTEROL LIPOSOMES

	[^{3}H]Thymidine			[^{14}C]Thymidine-monophosphate		
Sample	DPM	% of total	% Liposome Retention	DPM	% of total	% Liposome Retention
Total Added	28,600,000	100.	-	2,500,000	100.	-
MLV liposomes	23,740	0.11	100	24,100	1.1	100
Washed MLV	4,570	0.02	19	20,900	0.92	84
SUV liposomes	92,720	0.42	100	114,000	5.0	100
Washed SUV	19,510	0.09	21	98,500	4.3	86

When the above MLV and SUV were placed in an Amicon ultrafiltration cell and concentrated to a small volume, 86-87% of the [^{14}C]TMP was retained by the liposomes versus only 18-21% for [^{3}H]thymidine. Overall, only 0.02-.09% of the total [^{3}H]thymidine was retained versus 0.9-4.3% retained for [^{14}C]TMP. The fact that 50 times more [^{14}C]TMP was ultimately retained in liposomes indicates that [^{3}H]thymidine cannot be entrapped probably due to rapid diffusion across the lipid bilayer.

Labeled AZT-5'-monophosphate ([^{3}H]AZT-MP) was prepared according to the phosphorous oxychloride method of Toorchen and Topal (34). Twenty nanomoles of AZT (260 microcuries) was dried under nitrogen. Twenty microliters of trimethylphosphate and 4 microliters of triethylamine were added and the mixture cooled to -10°C. Four microliters of phosphorus oxychloride was added and the mixture was allowed to react at -10°C for 30 minutes. The reaction was stopped by addition of an equal volume of 0.5 M aqueous triethylamine. AZT was converted to AZT-MP in a yield of greater than 90 percent as judged by HPLC on Altex C18 Ultrosphere-ODS column.

The resulting [^{3}H]AZT-MP was encapsulated in liposomes in the same manner as the [^{14}C]thymidine phosphate and then tested for leakage against free labeled AZT ([^{3}H]AZT). The results of the tests are shown in Table 2. Only 0.2% of the [^{3}H]AZT was entrapped in liposomes versus 3.8% of the [^{3}H]AZT-MP. This indicates that [^{3}H]AZT, like [^{3}H]thymidine (Table 1), diffuses out of liposomes as they are being isolated on the Sepharose 4B column. In contrast, [^{3}H]AZT-MP was entrapped readily; 3.8% of the total was recovered in the liposomal fraction, a 19 fold increase over the percent encapsulation of [^{3}H]AZT.

TABLE 2

RETENTION OF [^{3}H]AZT AND [^{3}H]AZT-5'-MONOPHOSPHATE IN PC/CHOLESTEROL LIPOSOMES

	[^{3}H]AZT			[^{3}H]AZT-MP		
Sample	DPM	% of total	% Liposome Retention	DPM	% of total	% Liposom Retentio
Total [^{3}H]AZT	15,320,000	100.	-	13,200,000	100.	-
Liposome fraction	30,390	0.20	100	497,000	3.8	100
Amicon retentate #1	24,485	0.16	82	493,340	3.7	99
Amicon retentate #2	20,473	0.13	67	493,100	3.7	99
Amicon retentate #3	18,617	0.12	61	490,400	3.7	99
Liposomes, 20hr/4°C	13,144	0.09	43	-	-	-

The effect of liposomal AZT monophosphate on HIV replication in MT-2 and U937 cells and human macrophages in culture was tested as follows:

A thin film of 27 mg cholesterol and 110 mg egg phosphatidylcholine was prepared by rotary evaporation in vacuo and 1 ml of RPMI medium containing 60 nmol of AZT-MP and 6 uCi [^{3}H]AZT-MP was added and the mixture was shaken at 20^{0}C. for 20 min followed by 10 cycles of vortexing to produce MLV containing [^{3}H]AZT-MP.

Using a Lipex Extruder (Lipex Biomembranes, Inc., Vancouver, B.C.), small unilamellar vesicles of 100 nanometer diameter (EV 100) were prepared by the method of Mayer et al (36). This procedure is based on extrusion of large multilamellar vesicles through two stacked polycarbonate filters (Nucleopore, Pleasanton, CA).

The resulting liposome preparation was applied to a 1x15 cm column of Sepharose 4B and eluted with RPMI buffer. EV 100 liposomes containing [^{3}H]AZT-MP eluted at the void volume while free [^{3}H]-drug was collected in the column salt volume. Under these conditions, 14.3% of the [^{3}H]AZT-MP was trapped. Higher encapsulation efficiencies can be obtained by using more phospholipid per unit volume of buffer and by repeated freezing and thawing (36).

EV 100 liposomes containing [^{3}H]AZT-MP were added in various concentration to MT-2 and U937 cells growing in ELISA plates; to other wells were added AZT-MP and AZT. After 3 days the MT-2 cells (6x10^5 cells/well) were examined for cytopathic effects (CPE) and the results of CPE grading are shown in Table 3.

TABLE 3

Experiment E725

EFFECT OF LIPOSOMAL AZT-MP ON

HIV REPLICATION IN MT-2 CELLS. DAY 7

rmol drug/well	CPE Free AZT	CPE AZT-MP Liposome A	CPE AZT-MP Liposome B
2	0;0	0;0	0;0
0.2	0;0	0;0	0;0
0.02	4+4+	3+;0	0;0
0.002	4+;4	4+;4+	0;0
0.002	4+;4+	4;4+	4+;4+

Control, no drug, 4+4+; CPE,cytopathic effects. Liposomes containing [^{3}H]AZT-monophosphate were prepared in RPMI medium by the method of Mayer et al (36) and the liposomal AZT-MP encapsulated was determined by the ^{3}H content of the pooled liposomal peak. Liposomes containing [^{3}H]-AZT-MP were added to MT-2 cells infected with HIV in a final volume of 0.200 ml of RPMI medium containing 10% fetal calf serum. Liposome A consisted of 67 mole % egg phosphatidylcholine and 33 mole % cholesterol and Liposome B was made up of 6.6 mole % psychosine and 60 mole % egg phosphatidylcholine and 33.3 mole % cholesterol. Grading of CPD was done as described by Haertle et al (44). In this system 1+ corresponds to 1 to 3 syncytia (giant cells) per well. 2+ corresponds to 3-10 syncytia per well and up to 20% cell death; 3+ corresponds to 10-30 syncytia per well

and 20-70% drop in cell viability; 4+ corresponds to more than 30 syncytia per well and at least a 70% fall in viable cell count (44).

Similarly, $6x10^5$ U937 cells growing in ELISA plates were inoculated with HIV. AZT, AZT-MP and liposomal AZT-MP were added to the culture medium as noted above. After 4 days of growth the cell supernatants were removed, diluted 1:100 and assayed for HIV gp24 using the Dupont ELISA System (NEK-041). The results are shown in Table 4.

TABLE 4

EFFECT OF LIPOSOMAL AZT-MP ON HIV REPLICATION IN U937 CELLS

umol lipid/well	nmol drug/well	gp24 Antigen, pg/ml Free AZT	AZT-MP Liposome A	AZT-MP Liposome B
2.72	2.0	1050	30	30
0.86	0.63	1200	30	30
0.27	0.20	3450	30	30
0.086	0.063	2850	30	30
0.027	0.020	3500	850	30
0.0086	0.0063	2800	2,100	1,600

Liposomes containing [^{3}H]AZT-MP were prepared as in Table 1 and added to U937 cells infected with HIV in 0.200 ml of RPMI containing 10% fetal calf serum. Liposome A consisted of 67 mole % egg phosphatidylcholine and 33 mole % cholesterol and liposome B contained 6.6 mole % psychosine and 60 mole % egg

phosphatidylcholine and 33 mole % cholesterol. Results are mean of 2 replicates.

The effect of liposomal AZT-monophosphate on cultured human macrophages was also determined following the procedures used for MT-2 cells and U937 cells. The results are set forth in Table 5.

TABLE 5

EFFECT OF LIPOSOMAL AZT-MP ON HIV REPLICATION IN CULTURED HUMAN MACROPHAGES

		gp24 Antigen, pg/ml		
umol lipid/well	nmol drug/well	Free AZT	AZT-MP Liposome A	AZT-MP Liposome B
-	20.0	2016	n.d.	n.d.
-	6.3	1844	n.d.	n.d.
2.72	2.0	2524	30	30
0.86	0.63	2480	30	30
0.27	0.20	2116	30	160
0.086	0.063	2172	30	30
0.027	0.020	n.d.	30	220
0.0086	0.006	n.d.	300	648

n.d. = not determined. Liposome of egg phosphatidylcholine containing [^{3}H]AZT-MP in the indicated concentration were prepared as in Table 1 and added to human macrophages. After 3 days in culture the supernatants were assayed for gp24. Liposome A consisted of 67 mole % egg phosphatidylcholine and 33 mole % cholesterol and liposome B contained 6.6 mole % psychosine and 60 mole % egg phosphatidylcholine and 33 mole % cholesterol.

Additional examples of practice are as follows:

Liposomes of egg phosphatidylcholine/cholesterol (2/1) (100 nanometer diameter) containing [^{3}H]AZT-MP were prepared in RPMI medium by the extrusion method of

Mayer et al (36), and the amount of [^{3}H]AZT-MP encapsulated was determined by ^{3}H counts in the voiding peak obtained by gel permeating chromatography using Sepharose 4B. Varying amounts of AZT-MP were added and the effect determined on cells infected with HIV by measuring production of HIV antigen gp24 using the DuPont Elisa assay kit. Two liposome types were prepared -- liposome A and liposome B. Liposome A consisted of 67 mole % egg phosphatidylcholine and 33 mole % cholesterol and liposome B contained 6.6 mole % psychosine and 60 mole % egg phosphatidylcholine and 33 mole % cholesterol. The results of the tests are shown in Table 6.

TABLE 6

EFFECT OF LIPOSOMAL AZT-MP ON HIV REPLICATION IN U937 CELLS

umol PC/well	nmol drug/well	gp24 Antigen, pg/ml Free AZT	AZT-MP Liposome A	AZT-MP Liposome B
4.6	2.0	2,740	60	60
0.46	0.2	3,120	78	60
0.046	0.02	5,060	2,140	1,353
0.0046	0.002	4,460	2,120	13,290
0.00046	0.0002	10,910	13,100	13,980
0	0	(15,420)	-	

The liposomal AZT-MP prepared for testing against U937 cells as set forth in Table 6 was also used against HIV-infected human macrophages. The tests were conducted in the same manner as previously described for Table 6 and the results are set forth in Table 7.

TABLE 7

EFFECT OF LIPOSOMAL AZT-MP ON
HIV REPLICATION IN HUMAN MACROPHAGES

umol PC/well	nmol drug/well	gp24 Antigen, pg/ml		
		Free AZT	AZT-MP Liposome A	AZT-MP Liposome B
4.6	2.0	2,016	30	30
0.46	0.2	1,872	30	30
0.046	0.02	2,268	30	49
0.0046	0.002	2,132	2,084	1,632
0.00046	0.0002	2,248	1,732	1,316
0.000046	0.00002	2,800	1,448	1,744
0	0	2,276	-	

Having thus described exemplary embodiments of the present invention, it should be noted by those skilled in the art that the within disclosures are exemplary only and that various other alternatives, adaptations and modifications may be made within the scope of the present invention. Accordingly, the present invention is not limited to the specific embodiments as illustrated herein, but is limited only by the following claims.

BIBLIOGRAPHY

1. Hirsch, M.S., J.C. Kaplan. 1987. Antiviral Therapy. Scientific American (April 1987) pages 76-85.

2. Gallo, R.C. 1987. The AIDS Virus. Scientific American (January 1987) pages 47-56.

3. Dalgleish, A.G., P.C.L. Beverley, P.R. Claphan, D.H. Crawford, M.F. Greaves, and R.A. Weiss. 1984. The CD4 (T4) antigen is an essential component of the receptor for the AIDS retrovirus. Nature 312:763.

4. Klatzman, D., E. Champagne, S. Chamaaret, J. Gruest, D. Guetard, T. Hercend, J.-C. Gluckman, and L. Montagnier. 1984. T-lymphocyte T4 molecule behaves as the receptor for human retrovirus LAV. Nature 312:767.

5. Maddon, P.J., A.G. Dalgleish, J.S. McDouglal, P.R. Clapham, R.A. Weiss, and R. Axel. 1986. The T4 gene enclodes the AIDS virus receptor and is expressed in the immune system and the brain. Cell 47:333.

6. Lifson, J.D., M.B. Feinberg, G.R. Reyes, L. Rabin, B. Banapour, S. Chakrabarti, B. Moss, F. Wong-Staal, K.S. Steimer, and E.G. Engleman. 1986. Induction of CD4-dependent cell fusion by the HTLV-III/LAV envelope glycoprotein. Nature 323:725.

7. Yoffe, B., D. Lewis, B. Petrie, C. Noonan, J. Melnick, F.B. Hollinger. 1987. Fusion as a meadiator of cytolysis in mixtures of uninfected CD4+ lymphocytes and cells infected by human immunodeficiency virus. Proc. Natl. Acad. Sci. 84:1429.

8. Ho, D.D., T.R. Rota, and M.S. Hirsch. 1986. Infection of monocyte/macrophages by human T lymphotropic virus type III. J. Clin. Invest. 77:1712.

9. Chayt, K.S., M.E. Harper, L.M. Marselle, E.B. Lewin, R.M. Rose, J.M. Oleske, L.G. Epstein, F. Wong-Staal, and R.C. Gallo. 1986. Detection of HTLV-III RNA in lungs of patients with AIDS and pulmonary involvement. JAMA 256:2356.

10. Tschachler, E., V. Groh, M. Popovic, D.L. Mann, K. Konrad, B. Safai, L. Eron, F. diMarzo Veronese, K. Wolff and G. Stingl. 1987. Epidermal Langerhans cells - a target for HTLV-III/LAV infection. J. Invest. Dermatol. 88:233.

11. Mitsuya, H., K.J. Weinhold, P.A. Furman, M.H. St. Clair, S. Nusinoff Lehrman, R.C. Gallo, D. Bolognesi, D.W. Barry and S. Broder. 1985. 3'-azido-3'-deoxythymidine (BW A509U): An antiviral agent that inhibits the infectivity and cytopathic effect of human T-lymphotropic virus type III/lymphadenopathy-associated virus in vitro. Proc. Natl. Acad. Sci. 82:7096.

12. Mitsuya, H. and S. Broder. 1986. Inhibition of the in vitro infectivity and cytopathic effect of human T-lymphotrophic virus type III/lymphadenopathy-associated virus (HTLV-III/LAV) by 2',3'-dideoxynucleosides. Proc. Natl. Acad. Sci. 83:1911.

13. Furman, P.A., J.A. Fyfe, M.H. St. Clair, K. Weinhold, J.L. Rideout, G.A. Freeman, S. Nusinoff Lehrman, D.P. Bolognesi, S. Broder, H. Mitsuya, and D.W. Barry. 1986. Phosphorylation of 3'-azido-3'-deoxythymidine and selective interaction of the 5'-triphosphate with human immunodeficiency virus reverse transcriptase. Proc. Natl. Acad. Sci. 83:8333.

14. Cooney, D.A., M. Dalal, H. Mitsuya, J.B. McMahon, M. Nadkarni, J. Balzarini, S. Broder, and D.G. Johns. 1986. Initial studies on the cellular pharmacology of 2',3'-dideoxycytidine, an inhibitor

of HTLV-III infectivity. Biochem. Pharmacol. 35:2065.

15. Yarchoan, R.Y., R.W. Klecker, K.J. Weinhold, P.D. Markham, H.K. Lyerly, D.T. Durack, E. Gelmann, S. Nusinoff Lehrman, R.M. Blum, D.W. Barry, G.M. Shearer, M.A. Fischl, H. Mitsuya, R.C. Gallo, J.M. Collins, D.P. Bolognesi, C.E. Myers, S. Broder. 1986. Administration of 3'-azido-3'-deoxythymidine, an inhibitor of HTLV-III/LAV replication, to patients with AIDS or AIDS-related complex. Lancet 8481:575.

16. Norley, S.G., L. Huang and B.T. Rouse, 1987. Targeting of Drug Loaded Immunoliposomes to Herpes Simplex Virus Infected Corneal Cells: An Effective Means of Inhibiting Virus Replication in Vitro. Journal of Immunology. Vol. 136, No. 2: 681-685.

17. Kond, M., Alving, C.R., Rill, W.L., Swartz, G.M. and Cannonico, PP.G. 1985. Enhanced Efficacy of Liposome-encapsulated ribavirin against Rift Valley fever virus infection in mice. Antimicrob Agents Chemother. 27: 903-907.

18. Bangham, A.D., Standish, M.M. and Watkins, J.C. (1965) J. Mol. Biol., 13:238-252.

19. Gregoriadis, G. (1976) New Eng. J. Med., 295:704-710 and 765-770.

20. Post, G., Kirsch, R. and Koestler, T. (1984) in Liposome Technology, Vol. III, G. Gregoriadis, Ed., CRC Press, Boca Raton, p.1-28.

21. Scherphof, G. (1986) in Lipids and Membranes, Past, Present and Future, Op den Kamp J., Roelofsen, B. and Wirtz, K.W.A., eds., Elsevier North Holland, Amsterdam, p 113-136.

22. Ostro, M. (1987) Sci. Am. 256:103-111.

23. Black, C.D.V., Watson, G.J. and Ward, R.J. (1977) Trans. Roy. Soc. Trop. Med. Hyg. 71:550-552

24. Alving, C.R., Steck, E.A., Chapman, W.L. Waits,

V.B., Hendricks, L.D., Swartz, G.M., Hanson, W.L. (1978) Proc. Natl. Acad. Sci. USA, 75:2959-2963.

25. Lopez-Berestein, G. (1986) Ann. Int. Med., 105:130-131.

26. Shirkhoda, A., Lopez-Berestein, G., Holbert, J.M. and Luna, M.A. (1986) Radiol., 159:349-353.

27. Herman, E.H., Rahman, A. Ferrans, V.J., Vick, J.A., and Schein, P.S. (1983) Cancer Res., 43:5427-5432.

28. Levy, J., et al. (1985) Ann. Int. Med. 103:694-699.

29. Salahuddin, S.Z., Rose, R.M., Groopman, J.E., Markham, P.D., and Gallo R.C. (1985) Blood 68:281-284.

30. Koenig, S., Gendelman, H.E., Orenstein, J.M., Dalcanto, M.C., Pezeshkpur, G.H., Yungbluth, M., Janotta, F., Aksmit, A., Martin, M.A. and Fauci, A.S. (1986) Science, 233: 1089-1093.

31. McDougal, J.S., Kennedy, M.S., Sligh, J.M., Cort, S.P., Mawle, A. and Nicholson, J.K.A. (1986) Science 231:382-385.

32. Kaddon, P.J. Dalgleish, A.G., McDouglas, J.S. Clapham, P.R., Weiss, R.A. and Axel, R. (1986) Cell 47:333-348.

33. Yarchoan, R., Weinhold, K.J. Lyerly, H.K. Gelman, E., Blum, R.M., Shearer, G.M., Mitsuya, H., Collins, J.M., Myers, C.E., Klecker, R.W., Markham, P.D., Durack, D.T., Lehrman, S.N., Barry, D.W., Fischl, M.A., Bolognesi, D.P., and Broder, S. (1986) Lancet, March 15, 575-580.

34. Toorchen, D. and Topal, M.D. (1983) Carcinogenesis 4:1591-1597.

35. Fukunaga, M., Miller, M.M., Hostetler, K.Y. and Deftos, L.J. (1984) Endocrinol. 115:757-761.

36. Mayer, L.D., Hope, M.J. and Cullis, P.R. (1986) Biochim. Biophys. Acta. 858:161-168.

37. Fischl, M.A., Richman, D.D., Grieco, M.H., et al, (1987) New Eng. J. Med., 317:185-191.

38. Richman, D.D., Fischl, M.A., Grieco, M.H., et al, (1987), New Eng. J. Med., 317:192-197.

39. Olson, F., Hunt, C.A., Szoka, F.C., Vail, W.J. and Papahadjopoulos, D. (1979) Biochim. Biophys. Acta. 557:9-23.

40. Szoka, F., Jr. and Papahadjopoulos, D. (1978) Proc. Nat. Acad. Sci. 75:4194-4198.

41. Mayhew, E., Lazo, R., Vail, W.J., King, J., Green, A.M. (1984) Biochim. Biophys. Acta 775:169-175.

42. Kim, S., Turker, M., Chi, E., et al (1978) Biochim. Biophys. Acta. 728:339-348.

43. Richman, D.D., Kornbluth, R.S. and Carson, D.A. (1987) J. Exp. Med., 166:1144-1149.

44. Haertle, T., Carrera, C.J., McDougal, J.S., Sowers, L.C., Richman, D.D. and Carson D.A. (1987) J. Biol. Chem., in press.

What is claimed is:

1. A composition for use in treating acquired immune deficiency syndrome and related retroviral infections comprising a phosphorylated nucleoside analogue which is encapsulated in a liposome and a pharmaceutically acceptable carrier therefor.

2. A composition according to claim 1 wherein said nucleoside analogue is selected from the group consisting of azidothymidine, dideoxycytidine, dideoxyadenosine and ribavirin.

3. A composition according to claim 2 wherein said phosphorylated nucleoside analogue is the 5'-monophosphate derivative of azidothymidine.

4. A composition according to claim 2 wherein said phosphorylated nucleoside analogue is the 5'-monophosphate derivative of dideoxyadenosine.

5. A composition according to claim 2 wherein said phosphorylated nucleoside analogue is the 5'-monophosphate derivative of dideoxycytidine.

6. A composition according to claim 2 wherein said phosphorylated nucleoside analogue is the 5'-monophosphate derivative of ribavirin.

7. A composition according to claim 1, wherein said liposome is made from at least one of the phospholipids selected from the group consisting of phosphatidylcholine, phosphatidylethanolamine, phosphatidylglycerol, sphingomyelin, phosphatidylserine, phosphatidylinositol, dilauroylphosphatidylcholine, dimyristoylphosphatidylcholine, dioleoylphosphatidylcholine,

dipalmitoylphosphatidylcholine, distearoylphosphatidylcholine, dilauroylphosphatidylethanolamine, dimyristoylphosphatidylcholine, dipalmitoylphosphatidylcholine, dioleoylphosphatidylethanolamine and distearoylphosphatidylethanolamine.

8. A composition according to claim 7 wherein said liposome further includes additives selected from the group consisting of cholesterol, glycolipids, cerebrosides, gangliosides, sphingolipids, glucopsychosine, and psychosine.

9. A composition according to claim 8 wherein said liposome consists essentially of from about 80-99 mole percent egg phosphatidylcholine and from about 1 to 20 mole percent psychosine.

10. A composition according to claim 9 wherein said phosphorylated nucleoside analogue is the 5'-monophosphate derivative of azidothymidine.

11. A composition according to claim 1 wherein said pharmaceutically acceptable carrier is an aqueous buffer.

12. A method for treating acquired immune deficiency syndrome and related retroviral infections in mammals comprising the step of administering to said mammal a pharmaceutically acceptable dose of a composition comprising a phosphorylated nucleoside analogue which is encapsulated in a liposome and a pharmaceutically acceptable carrier therefor.

13. A method according to claim 12 wherein said nucleoside analogue is selected from the group consisting of azidothymidine, dideoxycytidine, dideoxyadenosine

and ribavirin.

14. A method according to claim 13 wherein said phosphorylated nucleoside analogue is the 5'-monophosphate derivative of azidothymidine.

15. A method according to claim 13 wherein said phosphorylated nucleoside analogue is the 5'-monophosphate derivative of dideoxyadenosine.

16. A method according to claim 13 wherein said phosphorylated nucleoside analogue is the 5' derivative of dideoxycytidine.

17. A method according to claim 13 wherein said phosphorylated nucleoside analogue is the 5'-monophosphate derivative of ribavirin.

18. A method according to claim 12 wherein said liposome is made from phospholipids selected from the group consisting of phosphatidylcholine, phosphatidylethanolamine, phosphatidylglycerol, sphingomyelin, phosphatidylserine, phosphatidylinositol, dilauroylphosphatidylcholine, dimyristoylphosphatidylcholine, dioleoylphosphatidylcholine, dipalmitoylphosphatidylcholine, distearoylphosphatidylcholine, dilauroylphosphatidylethanolamine, dimyristoylphosphatidylcholine, dipalmitoylphosphatidylcholine, dioleoylphosphatidylethanolamine and distearoylphosphatidylethanolamine.

19. A method according to claim 18 wherein said liposome further includes an additives selected from the group consisting of cholesterol, glycolipids, cerebrosides, gangliosides, sphingolipids, glucopsychosine, and psychosine.

20. A method according to claim 19 wherein said liposome consists essentially of from about 80-99 mole percent egg phosphatidylcholine and from about 1 to 20 mole percent psychosine.

21. A method according to claim 20 wherein said phosphorylated nucleoside analogue is the 5'-monophosphate derivative of azidothymidine.

22. A method according to claim 12 wherein said pharmaceutically acceptable carrier is an aqueous buffer.

23. A method according to claim 12 wherein said composition is administered intravenously to said mammal.

24. A composition according to claim 7 wherein the concentration of the phosphorylated nucleoside analogue entrapped within said liposome is between about 0.001 mM to 300 mM.

25. A method for preparing a medicament comprising the steps of encapsulating a phosphorylated nucleoside analogue in a liposome to form an encapsulated phosphorylated nucleoside analogue and combining said encapsulated phosphorylated nucleoside analogue with a pharmaceutically acceptable carrier therefor.

26. A method for preparing a medicament according to claim 25 wherein said phosphorylated nucleoside analogue is selected from the group consisting of azidothymidine, dideoxycytidine, dideoxyadenosine and ribavirin.

27. A method for preparing a medicament according

to claim 25 wherein said phosphorylated nucleoside analogue is prepared by reacting a nucleoside analogue with phosphorous oxychloride in the presence of trimethylphosphate and triethylamine.

28. A method for preparing a medicament according to claim 26 wherein said phosphorylated nucleoside analogue is the 5'-monophosphate derivative of azidothymidine.

29. A method for preparing a medicament according to claim 25 wherein said liposome is made from a phospholipid selected from the group consisting of phosphatidylcholine, phosphatidylethanolamine, phosphatidylglycerol, sphingomyelin, phosphatidylserine, phosphatidylinositol, dilauroylphosphatidylcholine, dimyristoylphosphatidylcholine, dioleoylphosphatidylcholine, dipalmitoylphosphatidylcholine, distearoylphosphatidylcholine, dilauroylphosphatidylethanolamine, dimyristoylphosphatidylcholine, dipalmitoylphosphatidylcholine, dioleoylphosphatidylethanolamine and distearoylphosphatidylethanolamine.

30. A composition according to claim 1 wherein said nucleoside analogue is a diphosphate derivative.

31. A composition according to claim 1 wherein said nucleoside analogue is a triphosphate derivative.

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US88/03210

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

INT.CL.(4):A61K 9/66, 37/22, 45/05; B01J 13/02.
U.S.CL. 264/4.1, 4.3; 424/450; 428/402.2; 436/829; 514/885.

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
U.S.CL.	264/4.1, 4.3; 424/450; 428/402.2; 436/829; 514/885.

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category *	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
Y	US,A, 4,235,871 PAPAHADJOPOULOS ET AL. PUBLISHED 25 NOVEMBER 1980 SEE EXAMPLES 1, 2 AND 9; AND COL. 6, LINES 31-35.	1-31
Y	BIOCHEMICAL PHARMACOLOGY VOL. 35, NO. 13 PUBLISHED 1986, COONEY ET AL "INITIAL STUDIES ON THE CELLULAR PHARMACOLOGY OF 2', 3'-DIDEOXYCITIDINE, AN INHIBITOR OF HTLV-III ACTIVITY", PAGES 2065-2068.	1,2,5,7-9, 11-13,16,18-20, 22-27,29-31
Y	PROC. NATL. ACAD. SCI. USA, VOL. 83 PUBLISHED NOVEMBER 1986, FURMAN ET AL. "PHOSPHORYLATION OF 3' - AZIDO--3'- DEOXYTHYMIDINE AND SELECTIVE INTERACTION OF THE 5' - TRIPHOSPHATE WITH HUMAN IMMUNODEFICIENCY VIRUS REVERSE TRANSCRIPTASE", PAGES 8333-8337.	1-3,7-14, 18-31
Y,P	US,A, 4,704,357 MITSUYA ET AL. PUBLISHED 03 NOVEMBER 1987, SEE EXAMPLE 3 AND COL. 1, LINE 5-COL. 2, LINE 2.	1,2,4,7-9, 11-13,15,18-20, 22-27,29-31
Y	ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, VOL. 27, NO. 6 PUBLISHED JUNE 1985. KENDE ET AL. "ENHANCED EFFICACY OF LIPOSOME-ENCAPSULATED RIBAVIRIN AGAINST RIFT VALLEY FEVER VIRUS INFECTION IN MICE", PAGES 903-907.	1,2,6-9,11-13, 17-20,22-27. 29-31

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
01 DECEMBER 1988	05 JAN 1989
International Searching Authority	**Signature of Authorized Officer**
ISA/US	Richard D. Lovering RICHARD D. LOVERING

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
Y	US,A, 4,544,552 FRAEFEL ET AL. PUBLISHED 01 OCTOBER 1985 SEE ABSTRACT; COL. 5, LINES 22-42; AND COL. 9, LINE 31.	9,10,20,21
X Y	NEW ANTI-RETROVIRAL AGENTS AND DELIVERY SYSTEM FOR THE SAME, (U.S.) DEPARTMENT OF HEALTH AND HUMAN SERVICES, WASHINGTON, D.C., PB87-224382 PUBLISHED 10 APRIL 1987, PAGES 1-21.	1-8,11-19,22-31 9,10,20,21
A	US,A, 4,291,024 TURCOTTE PUBLISHED 22 SEPTEMBER 1981.	1-31